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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
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LLC. Hatten-Heckman

Den Haag, den  
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**Sheet 2 of the certificate**  
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Peptides of Human Papilloma Virus for use in human T cell response inducing compositions

Field of the invention

The invention is concerned with novel peptides derived from Human Papilloma Virus proteins and their use in pharmaceutical compositions for a prophylactic or therapeutic treatment of human individuals against Human Papilloma Virus-related diseases such as cervical cancer.

Background of the invention

Human Papilloma Viruses (HPVs) are implicated in the etiology of cervical cancer, the fifth most common cancer worldwide and the second cause of cancer-related death in women. If other HPV-related cancers are counted also, up to 10% of the worldwide mortality due to cancer is linked to HPVs. HPVs are double stranded circular DNA viruses of about 8 kilobases. Until now more than 60 genotypes have been described of which several are associated with cancer.

HPV-DNA can be found in cervical dysplastic lesions and in cervical carcinomas in which the percentage of HPV positivity increases up to 99% when the lesions progress towards malignancy. The most important HPV types associated with cervical carcinoma are HPV16 and 18 of which HPV16 alone accounts for more than 50% of the HPV positive cervical carcinomas.

The DNAs of several HPVs have been sequenced. The DNA open reading frames can be divided into early regions (E) and late regions (L). The E regions are coding for proteins needed for virus replication and transformation. The L regions encode viral capsid proteins. The E6 and E7 proteins are involved in the pathogenesis of HPV-induced abnormal cell proliferation and these

genes are expressed in tissue or tumor cells obtained from cervical cancers associated with HPV infection.

In addition, the E6 and E7 genes of HPV16 and HPV18 are capable of inducing epithelial cell transformation in the cell culture without the presence of other HPV genes indicating that at least part of the stimulation of cell proliferation caused by HPV infection is due to the E6 and E7 viral proteins.

Cytotoxic T lymphocytes (CTL) are of crucial importance in the resistance against virus infections and the immune surveillance against virus-induced tumors (reviewed in 1). CTL specific for viruses or virus-induced tumors recognize short viral protein-derived peptides, of about 9 amino acids in length, that are bound to the antigen presenting groove of major histocompatibility complex (MHC) class I molecules (reviewed in 1). Recently in several virus systems vaccination with peptides recognized by antigen-specific CTL was shown to prevent lethal virus infections and to delay tumor growth in mice (reviewed in 1 and 2).

We have succeeded in the identification of viral peptides that bind to the groove of MHC class I molecules by using the antigen processing defective cell line 174.CEM T2 generated and provided by P. Cresswell (3). This cell line expresses the human MHC class I HLA-A2.1 and HLA-B5 alleles of which only the HLA-A2.1 molecules are expressed as partly empty and unstable molecules that can be stabilized on the cell surface with exogenously added peptides. If incubation with peptide results in an increase in the cell surface expression of this MHC molecule, this implies that the peptide binds to the groove of the HLA-A2.1 molecule and is therefore a possible candidate to be recognized by CTL. The HLA-A2.1 molecule is the most frequent HLA molecule present in the Western European Caucasoid population. About 50% of this population expresses this allele.

Using the amino acid sequence of the E6 and E7 proteins of HPV16 and HPV18 (4) we generated all possible nonapeptides (i.e. 9 amino acid long peptides) overspanning the entire E6 and E7 region. Every amino acid was used as a start amino acid for these 9-mer peptides. Every peptide was individually tested with respect

to its capacity to bind to the HLA-A2.1 molecule. With respect to HPV16, in total 10 peptides in the HPV16 E6 region and 8 in the HPV16 E7 region were identified to bind to the HLA-A2.1 molecule. With respect to HPV18, in total 9 peptides in the HPV18 E6 region and 5 in the HPV18 E7 region were identified to bind to the HLA-A2.1 molecule. This implies that all important candidate peptides of HPV16 and HPV18 for use as a vaccine in HLA-A2.1 positive humans have been identified.

The purpose of the present invention is therefore to provide specific synthetic peptides for prevention, prophylaxis, therapy and treatment of cervical carcinoma and/or adenoma and other HPV16- and/or HPV18-related diseases utilizing these synthetic peptides and pharmaceutical compositions containing the synthetic peptides.

#### Summary of the invention

The present invention provides specific peptides derived from the amino acid sequence of the E6 and E7 regions of HPV16 and HPV18 which, because of their capability to bind to the HLA-A2.1 protein, are candidate peptides to be included in human vaccines that can induce protective or therapeutic T cell responses against HPV16 and/or HPV18.

The novel peptides of the present invention are useful in pharmaceutical compositions, as screening tools and in the prevention, prophylaxis, therapy and treatment of HPV16- and/or HPV18-induced diseases or other conditions which would benefit from inhibition of HPV16 and/or HPV18 infection.

This invention provides a peptide comprising an amino acid sequence derived from a protein of human papilloma virus (HPV), wherein said amino acid sequence has the ability to bind to a human Major Histocompatibility Complex (MHC) Class I molecule.

In a preferred embodiment of the invention, said amino acid sequence is derived from protein E6 or E7 of HPV16 or from protein E6 or E7 of HPV18. Preferably, said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A2.1.

More specifically, this invention provides a peptide comprising an amino acid sequence derived from protein E6 or E7 of HPV16 or from protein E6 or E7 of HPV18, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A2.1 and is selected from the group consisting of:

1. KLPQLCTEL (residues 18-26 of HPV16 protein E6)
2. QLCTELQTT (residues 21-29 of HPV16 protein E6)
3. LCTELQTTI (residues 22-30 of HPV16 protein E6)
4. ELQTTIHDI (residues 25-33 of HPV16 protein E6)
5. LQTTIHDI (residues 26-34 of HPV16 protein E6)
6. TIHDIILEC (residues 29-37 of HPV16 protein E6)
7. IHDIILECV (residues 30-38 of HPV16 protein E6)
8. CVYCKQQLL (residues 37-45 of HPV16 protein E6)
9. KISEYRHYC (residues 79-87 of HPV16 protein E6)
10. PLCDLLIRC (residues 102-110 of HPV16 protein E6)
11. TLHEYMLDL (residues 7-15 of HPV16 protein E7)
12. YMLDLQPET (residues 11-19 of HPV16 protein E7)
13. MLDLQPETT (residues 12-20 of HPV16 protein E7)
14. RLCVQSTHV (residues 66-74 of HPV16 protein E7)
15. TLEDLLMGT (residues 78-86 of HPV16 protein E7)
16. LLMGTLGIV (residues 82-90 of HPV16 protein E7)
17. GTLGIVCPI (residues 85-93 of HPV16 protein E7)
18. TLGIVCPIC (residues 86-94 of HPV16 protein E7)
19. KLPDLCTEL (residues 13-21 of HPV18 protein E6)
20. SLQDIEITC (residues 24-32 of HPV18 protein E6)
21. LQDIEITCV (residues 25-33 of HPV18 protein E6)
22. EITCVYCKT (residues 29-37 of HPV18 protein E6)
23. KTVLELTEV (residues 36-44 of HPV18 protein E6)
24. ELTEVFEFA (residues 40-48 of HPV18 protein E6)
25. FAFKDLFVV (residues 47-55 of HPV18 protein E6)
26. DTLEKLTNT (residues 88-96 of HPV18 protein E6)
27. LTNTGLYNL (residues 93-101 of HPV18 protein E6)
28. TLQDIVLHL (residues 7-15 of HPV18 protein E7)
29. FQQLFLNTL (residues 86-94 of HPV18 protein E7)
30. QLFLNTLSF (residues 88-96 of HPV18 protein E7)

31. LFLNTLSFV (residues 89-97 of HPV18 protein E7)

32. LSFVCPWCA (residues 94-102 of HPV18 protein E7)

33. a fragment, homolog, isoform, derivative, genetic variant or conservative variant of any one of the amino acid sequences

5 Nos. 1-32 which has the ability to bind to human MHC Class I allele HLA-A2.1.

This invention further provides a pharmaceutical composition containing a prophylactically or therapeutically effective amount of a peptide according to the invention, and a pharmaceutically  
10 acceptable carrier, diluent, excipient or adjuvant. Preferably, said pharmaceutical composition contains a peptide according to the invention which is able to induce a T cell response effective against HPV.

In addition, this invention provides a method of prophylactic  
15 or therapeutic treatment of cervical carcinoma and other HPV-related diseases with a human individual, comprising administering to said human individual a prophylactically or therapeutically effective amount of a peptide according to the invention, more specifically an immunogenic form of a peptide according to the  
20 invention which is able to induce a T cell response effective against HPV.

#### Brief description of the drawings

25 Figure 1 gives the result of binding analyses of 240 HPV16 E6 and E7 nonapeptides. Background fluorescence level (without adding peptides) was set on an arbitrary mean fluorescence level of 70. Binding of a peptide was regarded positive when twice the level of background fluorescence was reached. The 18 binding  
30 peptides are numbered 1 to 18.

Figure 2 gives the result of binding analyses of 247 HPV18 E6 and E7 nonapeptides. Background fluorescence level (without adding peptides) was set on an arbitrary mean fluorescence level of 70. Binding of a peptide was regarded positive when twice the  
35 level of background fluorescence was reached. The 14 binding

peptides are numbered 1 to 14 (in the text, these HPV18 peptides are numbered 19 to 32).

#### Detailed description of the invention

5

The invention is directed to peptides comprising an amino acid sequence derived from a protein of HPV, wherein said amino acid sequence has the ability to bind to a human MHC Class I molecule. A most preferred embodiment of the invention concerns  
10 peptides comprising an amino acid sequence derived from protein E6 or E7 of HPV16 or HPV18, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A2.1. Specifically, such peptides comprise the following amino acid sequences derived from the noted regions of HPV16 and HPV18 (see  
15 Table I; the amino acids are identified by the one-letter code of amino acids).



TABLE I

Peptide		Amino acid sequence	HPV16/ HPV18	region
5	1	KLPQLCTEL	HPV16	E6 (residues 18 - 26)
	2	QLCTELQTT	HPV16	E6 (residues 21 - 29)
	3	LCTELQTTI	HPV16	E6 (residues 22 - 30)
	4	ELQTTIHDI	HPV16	E6 (residues 25 - 33)
	5	LQTTIHDI	HPV16	E6 (residues 26 - 34)
10	6	TIHDIILEC	HPV16	E6 (residues 29 - 37)
	7	IHDIILECV	HPV16	E6 (residues 30 - 38)
	8	CVYCKQQLL	HPV16	E6 (residues 37 - 45)
	9	KISEYRHYC	HPV16	E6 (residues 79 - 87)
	10	PLCDLLIRC	HPV16	E6 (residues 102-110)
15	11	TLHEYMLDL	HPV16	E7 (residues 7 - 15)
	12	YMLDLQPET	HPV16	E7 (residues 11 - 19)
	13	MLDLQPETT	HPV16	E7 (residues 12 - 20)
	14	RLCVQSTHV	HPV16	E7 (residues 66 - 74)
	15	TLEDLLMGT	HPV16	E7 (residues 78 - 86)
20	16	LLMGTLGIV	HPV16	E7 (residues 82 - 90)
	17	GTLGIVCPI	HPV16	E7 (residues 85 - 93)
	18	TLGIVCPIC	HPV16	E7 (residues 86 - 94)
	19	KLPDLCTEL	HPV18	E6 (residues 13 - 21)
	20	SLQDIEITC	HPV18	E6 (residues 24 - 32)
25	21	LQDIEITCV	HPV18	E6 (residues 25 - 33)
	22	EITCVYCKT	HPV18	E6 (residues 29 - 37)
	23	KTVLELTEV	HPV18	E6 (residues 36 - 44)
	24	ELTEVFEFA	HPV18	E6 (residues 40 - 48)
	25	FAFKDLFVV	HPV18	E6 (residues 47 - 55)
30	26	DTLEKLTNT	HPV18	E6 (residues 88 - 96)
	27	LTNTGLYNL	HPV18	E6 (residues 93 -101)
	28	TLQDIVLHL	HPV18	E7 (residues 7 - 15)
	29	FQQLFLNTL	HPV18	E7 (residues 86 - 94)
	30	QLFLNTLSF	HPV18	E7 (residues 88 - 96)
35	31	LFLNTLSFV	HPV18	E7 (residues 89 - 97)
	32	LSFVCPWCA	HPV18	E7 (residues 94 -102)

The data suggest that peptides 1-32 are single polypeptides of identified sequences. However, homologs, isoforms or genetic variants of these peptides 1-32 may exist within or outside the cellular environment. This invention encompasses all such

5 homologs, isoforms or genetic variants of peptides 1-32 provided that they bind to the HLA-A2.1 molecule. Polypeptides that are homologs of peptides 1-32 specifically include those having amino acid sequences which are at least about 40% conserved in relation to the amino acid sequence set forth in Table I, preferentially at  
10 least about 60% conserved, and more preferentially at least about 75% conserved.

It will be understood by one of ordinary skill in the art that other variants of peptides 1-32 are included within the scope of the present invention. This particularly includes any variants  
15 that differ from the synthesized peptides 1-32 only by conservative amino acid substitution. In particular, replacements of C (cysteine) by A (alanine), S (serine),  $\alpha$ -aminobutyric acid and others are included as it is known that cysteine-containing peptides are susceptible to (air) oxidation during synthesis and  
20 handling. Many such conservative amino acid substitutions are set forth as sets in (5).

Herein, peptides 1-32 or fragments thereof include any variation in the amino acid sequence, whether by conservative amino acid substitution, deletion, or other processes, provided  
25 that the polypeptides bind to the HLA-A2.1 molecule. The fragments of peptides 1-32 may be small peptides with sequences of as little as 5 or more amino acids, said sequences being those disclosed in Table I when said polypeptides bind to the HLA-A2.1 molecule.

Polypeptides larger than peptides 1-32 are especially  
30 included within the scope of the present invention when said polypeptides induce a HPV16- or HPV18-specific CTL response in HLA-A2.1 positive individuals and include a (partial) amino acid sequence as set forth in Table I, or conservative substitutions thereof. Such polypeptides may have a length up to about 30 amino  
35 acids, preferably up to about 27 amino acids.

This invention includes the use of polypeptides 1-32 generated by every means, whether genetic engineering, peptide synthesis with solid phase techniques or others. The foregoing peptides 1-32 may have various chemical modifications made at the terminal ends and still be within the scope the present invention. Also other chemical modifications are possible, particularly cyclic and dimeric configurations. The term "derivatives" intends to cover all such modified peptides.

The polypeptides of the present invention find utility for the treatment or prevention of diseases involving HPV16 or HPV18 such as genital warts, cervical cancer or others that are linked to HPV16 or HPV18.

For all applications the peptides are administered in an immunogenic form. Since the peptides are relatively short, this may necessitate conjugation with an immunogenicity conferring carrier material such as lipids or others or the use of adjuvants.

The magnitude of a prophylactic or a therapeutic dose of polypeptides 1-32 of this invention will, of course, vary with the group of patients (age, sex, weight, etcetera), the nature of the severity of the condition to be treated, the particular polypeptide of this invention and its route of administration. Any suitable route of administration may be employed to achieve an effective dosage of a polypeptide identified by this invention, as well as any dosage form well known in the art of pharmacy. In addition the polypeptides 1-32 may also be administered by controlled release means and/or delivery devices. They may also be administered in combination with other active substances, such as, in particular, T-cell activating agents like interleukine-2 etc.

The peptides of this invention may also be useful for other purposes, such as diagnostic use. For example, they may be used to check whether a vaccination with a peptide according to the invention has been successful. This may be done in vitro by testing whether said peptide is able to activate T cells of the vaccinated person.

In order to identify E6 and E7 region peptides of HPV16 and HPV18 that could bind to HLA-A2.1 molecules, the amino acid

sequences of E6 and E7 of HPV16 and HPV18 were examined (4). Every amino acid in the E6 and E7 region was used as the first amino acid of a 9 amino acid long peptide. In this way the entire E6 and E7 regions of HPV16 and HPV18 were covered. Nine amino acid long peptides were chosen because they fit the presently known rules for length of peptides that bind to the groove of HLA-A2.1 molecules (reviewed in 1). For practical reasons, in a first series of experiments alanine residues were used in the tested peptides instead of the cysteine residues occurring in the natural sequence. Thereafter, a second series of experiments was carried out with peptides containing the cysteine residues of the natural sequence.

Only the 32 peptides described in Table I (including those containing alanine residues instead of the cysteine residues) were able to significantly upregulate the expression of HLA-A2.1 molecules measured as mean HLA-A2.1 fluorescence of 174. CEM T2 cells indicating their binding to the HLA-A2.1 molecule as described in Example 2. None of the 222 + 233 other peptides were able to do this. The results of the fluorescence measurement are given in Table II and shown in Figures 1-2.

MF = Mean Fluorescence

$$FI = \text{Fluorescence Index} = \frac{(MF)_{\text{experiment}} - (MF)_{\text{blank}}}{(MF)_{\text{blank}}}$$

With background fluorescence level (without adding peptides) set on a Fluorescence Index level of 0, binding of a peptide was regarded positive when the level of fluorescence was  $\geq 0.5$ .

TABLE II

	Peptide	FI	MF	origin
5	1	1.6	168.6	HPV16 E6
	2	2.2	211.9	HPV16 E6
	3	0.7	113.6	HPV16 E6
	4	0.6	108.0	HPV16 E6
	5	1.6	170.4	HPV16 E6
10	6	1.8	184.5	HPV16 E6
	7	1.3	150.7	HPV16 E6
	8	2.0	195.0	HPV16 E6
	9	2.2	211.7	HPV16 E6
	10	2.1	204.8	HPV16 E6
15	11	1.2	144.5	HPV16 E7
	12	3.3	283.9	HPV16 E7
	13	2.3	217.6	HPV16 E7
	14	2.8	250.0	HPV16 E7
	15	1.8	182.8	HPV16 E7
20	16	0.8	118.8	HPV16 E7
	17	2.3	216.1	HPV16 E7
	18	2.5	227.8	HPV16 E7
	19	2.1	439.3	HPV18 E6
	20	0.7	243.9	HPV18 E6
25	21	1.3	327.8	HPV18 E6
	22	0.5	206.0	HPV18 E6
	23	2.3	467.5	HPV18 E6
	24	1.1	289.0	HPV18 E6
	25	2.3	462.9	HPV18 E6
30	26	0.7	238.2	HPV18 E6
	27	1.5	352.5	HPV18 E6
	28	2.7	519.6	HPV18 E7
	29	0.8	244.5	HPV18 E7
	30	0.8	258.2	HPV18 E7
35	31	1.6	360.3	HPV18 E7
	32	0.9	262.0	HPV18 E7

These experiments indicate that only a limited proportion of peptides (named 1-32) have the ability to bind to the HLA-A2.1 molecule and are therefore the only candidates of the HPV16 and HPV18 E regions to be recognized by human CTL because CTL  
5 recognize peptides only when bound to HLA molecules.

The following examples illustrate the present invention without limiting the same thereto.

#### EXAMPLE 1

##### Materials

##### Peptide synthesizer:

Abimed AMS 422 (Abimed Analysen-Technik GmbH, Langenfeld,  
15 Germany).

##### Synthesis polymer:

Tentagel S AC (0.17-0.24 meq/g, Rapp Polymere, Tübingen,  
Germany).

##### HPLC equipment:

20 The HPLC system used for analysis and purification of peptides consisted of: autosampler 2157, HPLC pump 2248, variable wavelength monitor VWM 2141, column oven 2155, low pressure mixer, all of Pharmacia Nederland B.V., Woerden, The Netherlands, a Star LC-20 dot matrix printer, Star  
25 Micronics Co., Ltd., all parts controlled by a Tandon PCAs1/386sx computer, Tandon Computer Benelux B.V., Amsterdam, The Netherlands.

##### Lyophilizer:

30 Virtis Centry, The Virtis Company, Inc., Gardiner (NY), USA, equipped with an Alcatel 350C vacuum pump, Alcatel CIT, Malakoff, France, connected to a Christ Alpha RVC vacuo-spin, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany.

##### Centrifuge:

35 MSE Mistral 6L, Beun de Ronde, Abcoude, The Netherlands.

## Mass spectrometer:

Bioion plasma desorption mass spectrometer (PDMS), Applied Biosystems, Inc., Foster City (CA), USA.

## Amino acid Analysis:

5 HP Aminoquant, Hewlett Packard, Amstelveen, The Netherlands.

## Chemicals:

All chemicals were used without further purification unless stated otherwise.

10 Fmoc (9-fluorenylmethyloxycarbonyl) amino acid were of the L-configuration, bearing the following side chain protecting groups: t-Bu (tert-butyl) for Asp, Glu, Tyr, Ser and Thr; Trt (trityl) for His, Asn and Gln; Pmc (2,2,5,7,8-penta-methylchroman-6-sulfonyl) for Arg; Boc (tert-butyloxy-carbonyl) for Lys, all Novasyn and purchased from Pharmacia  
15 Nederland B.V., Woerden, The Netherlands.

Piperidine was purchased from Aldrich Chemie Benelux N.V., Brussels, Belgium.

20 BOP (benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate) was obtained from Richelieu Biotechnologies, St-Hyacinthe, Canada.

N-methylmorpholin (NMM, Janssen Chimica, Tilburg, The Netherlands) was distilled from NaOH at atmospheric pressure before use.

25 N-methylpyrrolidone (NMP, Aldrich Chemie) was vacuum-distilled under a nitrogen atmosphere (b.p. 78-80°C, 18 mm Hg) before use.

Acetonitrile (HPLC-grade) was purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland.

30 Ether (Baker Analyzed grade), pentane (Baker grade) and acetic acid (Baker Analyzed grade) were purchased from J.T. Baker B.V., Deventer, The Netherlands.

Ethanethiol was obtained from Fluka Chemie, Brussels, Belgium.

35 Dichloromethane and N,N-dimethylacetamide (DMA) were purchased from Janssen Chimica, Tilburg, The Netherlands.

Trifluoroacetic acid (TFA, z.S. grade) was obtained from Merck-Schuchardt, Hohenbrunn, Germany.

Disposables:

Polypropylene reaction vessels containing a PTFE filter were purchased from Abimed Analysen-Technik GmbH, Langenfeld, Germany.

All other disposables used were made of polypropylene and obtained from Sarstedt B.V., Etten-Leur, The Netherlands.

Experimental conditions:

All experiments were performed at room temperature unless stated otherwise. All Fmoc protected aminoacids, synthesis polymers, peptides and TFA were stored at -20°C.

Peptide synthesis

Peptides were synthesized by solid phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422) (6, 7).

The peptides were made in various runs, in each of which 48 different peptides were synthesized simultaneously.

Tentagel S AC (8, 9), a graft polymer of polyethyleneglycol spacer arms on a polystyrene matrix, was used as a resin (40-60 mg per peptide, 10 µmol Fmoc amino acid loading).

Repetitive couplings were performed by adding a mixture of 90 µl 0.67 M BOP (10, 11) in NMP, 20 µl NMM in NMP 2/1 (v/v) and 100 µl of an 0.60 M solution of the appropriate Fmoc amino acid (12) in NMP (6-fold excess) to each reaction vessel. At 70% of the reaction time approximately 50 µl dichloromethane was added to each reaction vessel.

Fmoc-deprotection was performed by adding 3 times 0.8 ml of piperidine/DMA 1/4 (v/v) to each reaction vessel.

Coupling- and deprotection times were increased as the synthesis proceeded, starting with 30 min and 3 times 3 min respectively.

Washings after couplings and Fmoc-deprotections were done with 6 times 1.2 ml DMA. After the required sequence had been reached and the last Fmoc-protection was removed the peptidylresin



was washed extensively with DMA, dichloromethane, dichloromethane/ether 1/1 (v/v) and ether respectively, and dried.

#### Peptide cleavage and isolation

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Cleavage of the peptides from the resin and removal of the side chain protecting groups was performed by adding 6 times 200  $\mu$ l TFA/water 19/1 (v/v) at 5 min intervals to each reaction vessel, thus yielding free carboxylic peptides. For Trp containing

10

peptides TFA/water/ethanethiol 18/1/1 (v/v/v) was used.

Two hours after the first TFA addition the peptides were precipitated from the combined filtrates by addition of 10 ml ether/pentane 1/1 (v/v) and cooling to  $-20^{\circ}\text{C}$ . The peptides were isolated by centrifugation ( $-20^{\circ}\text{C}$ , 2500g, 10 min).

15

After treatment of the pellet with ether/pentane 1/1 (v/v) and isolation by the same centrifugation procedure, the peptides were dried at  $45^{\circ}\text{C}$  for 15 min.

Each of the peptides was dissolved in 2 ml water (or 2 ml 10 vol.% acetic acid), the solution frozen in liquid nitrogen for

20

3 min, and lyophilized while being centrifuged (1300 rpm, 8-16 h).

#### Analysis and purification

The purity of the peptides was determined by reversed phase HPLC; an aliquot of about 50 nmol was dissolved in 100  $\mu$ l 30 vol.% acetic acid. Of this solution 30  $\mu$ l was applied to an RP-HPLC system equipped with a ternary solvent system; A: water, B: acetonitrile, C: 2 vol.% TFA in water.

25

Gradient elution (1.0 ml/min) was performed from 90% A, 5% B, 5% C to 20% A, 75% B, 5% C in 30 min. Detection was at 214 nm.

30

Samples taken at random were analysed by mass spectrometry on a PDMS. The 31 binding peptides were all analysed by mass spectrometry on a PDMS and by quantitative amino acid analysis after hydrolysis on a HP Aminoquant. Of all analysed samples the

35

difference between calculated and measured masses was within the

experimental error (0.1%) as specified by the producer of the equipment used. All aminoacid compositions were as expected.

## EXAMPLE 2

### Peptides

Of all 240 HPV16 peptides and 247 HPV18 peptides that had been freeze dried, 5 mg was weighed and dissolved in 1 ml of distilled water adjusted with 5N NaOH to a pH of 12. Peptides that did not readily dissolve were treated with 150 µl of 100% acetic acid glacial (CH<sub>3</sub>COOH, Merck Darmstadt, Germany: 56-1000) after which the pH was neutralized to pH7 with 5N NaOH diluted in distilled water (Merck Darmstadt, Germany: 6498). Peptides which still did not dissolve were treated with 100 µl of 5N NaOH to pH 12 after which the pH was neutralized to pH 7 with 10% acetic acid glacial in distilled water. Of all peptides a dilution of 1 mg/ml in 0.9% NaCl was made.

### Cells

(174.CEM) T2 cells were cultured in Iscove's modified Dulbecco's medium (Biochrom KG Seromed Berlin, Germany: F0465) supplemented with 100IU/ml penicillin (Biocades Pharma, Leiderdorp, The Netherlands), 100 µg/ml kanamycin (Sigma St. Louis, USA:K-0254), 2mM glutamine (ICN Biomedicals Inc. Costa Mesa, CA, USA:15-801-55) and 10% fetal calf serum (FCS, Hyclone Laboratories Inc. Logan, Utah, USA:A-1115-L). Cells were cultured at a density of  $2.5 \times 10^5$ /ml during 3 days at 37°C, 5% CO<sub>2</sub> in humified air.

### Peptide binding

(174.CEM) T2 cells were washed twice in culture medium without FCS and put in serum free culture medium to a density of  $2 \times 10^6$  cells/ml. Of this suspension 40 µl was put into a V bottomed 96 well plate (Greiner GmbH, Frickenhausen, Germany: 651101)

together with 10  $\mu$ l of the individual peptide dilutions (of 1 mg/ml). The end concentration is 200  $\mu$ g/ml peptide with  $8 \times 10^4$  (174.CEM) T2 cells. This solution was gently agitated for 3 minutes after which an incubation time of 16 hours at 37°C, 5% CO<sub>2</sub> in humified air took place. Then cells were washed once with 100  $\mu$ l 0.9% NaCl, 0.5% bovine serum albumin (Sigma St. Louis, USA: A-7409), 0.02% NaN<sub>3</sub> (Merck Darmstadt, Germany:822335). After a centrifuge round of 1200 rpm the pellet was resuspended in 50  $\mu$ l of saturating amounts of HLA-A2.1 specific mouse monoclonal antibody BB7.2 for 30 minutes at 4°C. Then cells were washed twice and incubated for 30 minutes with F(ab)<sub>2</sub> fragments of goat anti-mouse IgG that had been conjugated with fluoresceine isothiocyanate (Tago Inc Burlingame, CA, USA: 4350) in a dilution of 1:40 and a total volume of 25  $\mu$ l.

After the last incubation, cells were washed twice and fluorescence was measured at 488 nanometer on a FACScan flowcytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The results are shown in fig. 1 (HPV16 peptides) and fig. 2 (HPV18 peptides), respectively.

The (174.CEM) T2 cell line expresses "empty" and unstable HLA-A2.1 molecules that can be stabilized when a peptide is binding to the peptide presenting groove of these molecules. A stabilized HLA-A2.1 molecule that will not easily degrade is the result of binding of an analyzed peptide. This leads to an increase in cell surface expression of the HLA-A2.1 molecule.

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## SEQUENCE LISTING

SEQ ID NO:1

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

LysLeuProGlnLeuCysThrGluLeu  
1 5 9

SEQ ID NO:2

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

GlnLeuCysThrGluLeuGlnThrThr  
1 5 9

SEQ ID NO:3

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

LeuCysThrGluLeuGlnThrThrIle  
1 5 9

SEQ ID NO:4

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

GluLeuGlnThrThrIleHisAspIle  
1 5 9

SEQ ID NO:5

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

LeuGlnThrThrIleHisAspIleIle  
1 5 9

SEQ ID NO:6

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

ThrIleHisAspIleIleLeuGluCys  
1 5 9

SEQ ID NO:7

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

IleHisAspIleIleLeuGluCysVal  
1 5 9

SEQ ID NO:8

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

CysValTyrCysLysGlnGlnLeuLeu  
1 5 9

SEQ ID NO:9

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

LysIleSerGluTyrArgHisTyrCys  
1 5 9

SEQ ID NO:10

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

ProLeuCysAspLeuLeuIleArgCys  
1 5 9

SEQ ID NO:11

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

ThrLeuHisGluTyrMetLeuAspLeu  
1 5 9

SEQ ID NO:12

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

TyrMetLeuAspLeuGlnProGluThr  
1 5 9

SEQ ID NO:13

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

MetLeuAspLeuGlnProGluThrThr  
1 5 9

SEQ ID NO:14

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

ArgLeuCysValGlnSerThrHisVal  
1 5 9

SEQ ID NO:15

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

ThrLeuGluAspLeuLeuMetGlyThr  
1 5 9

SEQ ID NO:16

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

LeuLeuMetGlyThrLeuGlyIleVal  
1 5 9

SEQ ID NO:17

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

GlyThrLeuGlyIleValCysProIle  
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SEQ ID NO:18

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

ThrLeuGlyIleValAlaProIleCys  
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SEQ ID NO:19

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

LysLeuProAspLeuCysThrGluLeu  
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SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

SerLeuGlnAspIleGluIleThrCys  
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SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

LeuGlnAspIleGluIleThrCysVal  
1 5 9

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SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

GluIleThrCysValTyrCysLysThr  
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SEQ ID NO:23

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

LysThrValLeuGluLeuThrGluVal  
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SEQ ID NO:24

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

GluLeuThrGluValPheGluPheAla  
1 5 9



SEQ ID NO:25

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

PheAlaPheLysAspLeuPheValVal  
1 5 9

SEQ ID NO:26

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

AspThrLeuGluLysLeuThrAsnThr  
1 5 9

SEQ ID NO:27

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

LeuThrAsnThrGlyLeuTyrAsnLeu  
1 5 9

SEQ ID NO:28

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

ThrLeuGlnAspIleValLeuHisLeu  
1 5 9

SEQ ID NO:29

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

PheGlnGlnLeuPheLeuAsnThrLeu  
1 5 9

SEQ ID NO:30

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

GlnLeuPheLeuAsnThrLeuSerPhe  
1 5 9

SEQ ID NO:31

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

LeuPheLeuAsnThrLeuSerPheVal  
1 5 9

SEQ ID NO:32

SEQUENCE TYPE: amino acid

SEQUENCE LENGTH: 9 amino acids

LeuSerPheValCysProTrpCysAla  
1 5 9

## CLAIMS

1. A peptide comprising an amino acid sequence derived from a protein of human papilloma virus (HPV), wherein said amino acid sequence has the ability to bind to a human Major Histocompatibility Complex (MHC) Class I molecule.
- 5 2. A peptide according to claim 1, wherein said amino acid sequence is derived from protein E6 or E7 of HPV16 or from protein E6 or E7 of HPV18.
3. A peptide according to claim 1, wherein said amino acid sequence has the ability to bind to human MHC Class I  
7 allele HLA-A2.1.
4. A peptide according to claim 2, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A2.1.
5. A peptide according to claim 4, wherein said amino acid  
15 sequence is selected from the group consisting of:
  1. KLPQLCTEL (residues 18-26 of HPV16 protein E6)
  2. QLCTELQTT (residues 21-29 of HPV16 protein E6)
  3. LCTELQTTI (residues 22-30 of HPV16 protein E6)
  4. ELQTTIHDI (residues 25-33 of HPV16 protein E6)
  - 20 5. LQTTIHDI (residues 26-34 of HPV16 protein E6)
  6. TIHDIILEC (residues 29-37 of HPV16 protein E6)
  7. IHDIILECV (residues 30-38 of HPV16 protein E6)
  8. CVYCKQQLL (residues 37-45 of HPV16 protein E6)
  9. KISEYRHYC (residues 79-87 of HPV16 protein E6)
  - 25 10. PLCDLLIRC (residues 102-110 of HPV16 protein E6)
  11. TLHEYMLDL (residues 7-15 of HPV16 protein E7)
  12. YMLDLQPET (residues 11-19 of HPV16 protein E7)
  13. MLDLQPETT (residues 12-20 of HPV16 protein E7)
  14. RLCVQSTHV (residues 66-74 of HPV16 protein E7)
  - 30 15. TLEDLLMGT (residues 78-86 of HPV16 protein E7)
  16. LLMGTGLGIV (residues 82-90 of HPV16 protein E7)
  17. GTLGIVCPI (residues 85-93 of HPV16 protein E7)
  18. TLGIVCPIC (residues 86-94 of HPV16 protein E7)

19. KLPDLCTEL (residues 13-21 of HPV18 protein E6)
  20. SLQDIEITC (residues 24-32 of HPV18 protein E6)
  21. LQDIEITCV (residues 25-33 of HPV18 protein E6)
  22. EITCVYCKT (residues 29-37 of HPV18 protein E6)
  - 5 23. KTVLELTEV (residues 36-44 of HPV18 protein E6)
  24. ELTEVFEEFA (residues 40-48 of HPV18 protein E6)
  25. FAFKDLFVV (residues 47-55 of HPV18 protein E6)
  26. DTLEKLTNT (residues 88-96 of HPV18 protein E6)
  27. LTNTGLYNL (residues 93-101 of HPV18 protein E6)
  - 10 28. TLQDIVLHL (residues 7-15 of HPV18 protein E7)
  29. FQQLFLNTL (residues 86-94 of HPV18 protein E7)
  30. QLFLNTLSF (residues 88-96 of HPV18 protein E7)
  31. LFLNTLSFV (residues 89-97 of HPV18 protein E7)
  32. LSFVCPWCA (residues 94-102 of HPV18 protein E7)
  - 15 33. a fragment, homolog, isoform, derivative, genetic variant or conservative variant of any one of the amino acid sequences Nos. 1-32 which has the ability to bind to human MHC Class I allele HLA-A2.1.
6. A pharmaceutical composition containing a
    - 20 prophylactically or therapeutically effective amount of a peptide according to any one of the claims 1-5, and a pharmaceutically acceptable carrier, diluent, excipient or adjuvant.
  7. A pharmaceutical composition containing a
    - 25 prophylactically or therapeutically effective amount of a peptide according to any one of the claims 1-5 which is able to induce a T cell response effective against HPV, and a pharmaceutically acceptable carrier, diluent, excipient or adjuvant.
  - 30 8. A method of prophylactic or therapeutic treatment of cervical carcinoma and other HPV-related diseases with a human individual, comprising administering to said human individual a prophylactically or therapeutically effective amount of a peptide according to any one of the claims 1-5.
  - 35 9. A method of prophylactic or therapeutic treatment of cervical carcinoma and other HPV-related diseases with a human

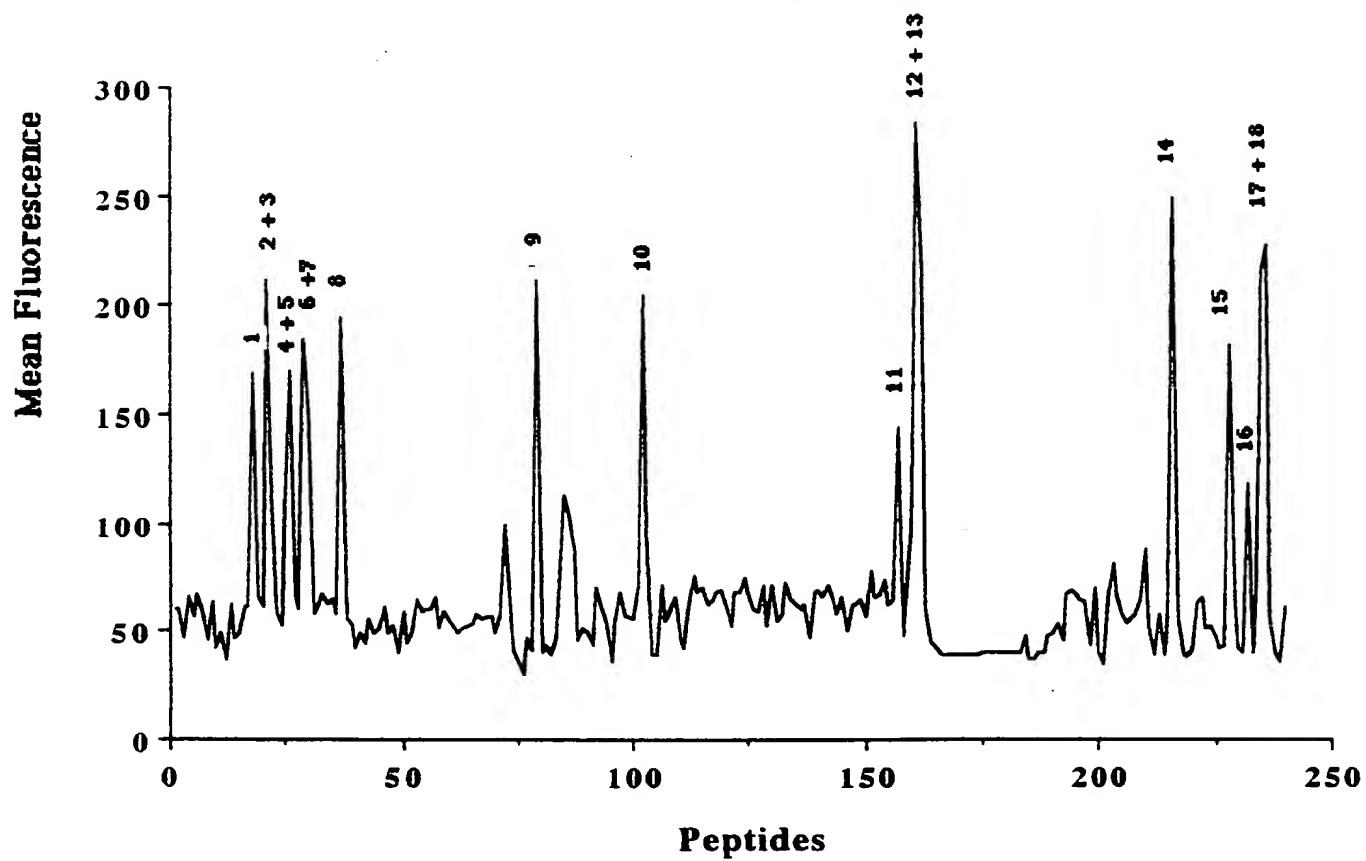
individual, comprising administering to said human individual a prophylactically or therapeutically effective amount of an immunogenic form of a peptide according to any one of the claims 1-5 which is able to induce a T cell response effective against

5 HPV.

## ABSTRACT

Peptides of Human Papilloma Virus for use in human T cell response inducing compositions

A peptide comprising an amino acid sequence derived from a human papilloma virus (HPV) protein, wherein said amino acid sequence has the ability to bind to a human Major Histocompatibility Complex Class I molecule. Its use in prophylactic or therapeutic treatment of cervical carcinoma and other HPV-related diseases.

**Binding of HPV16 E6/7 peptides to HLA-A2.1****FIG. 1**

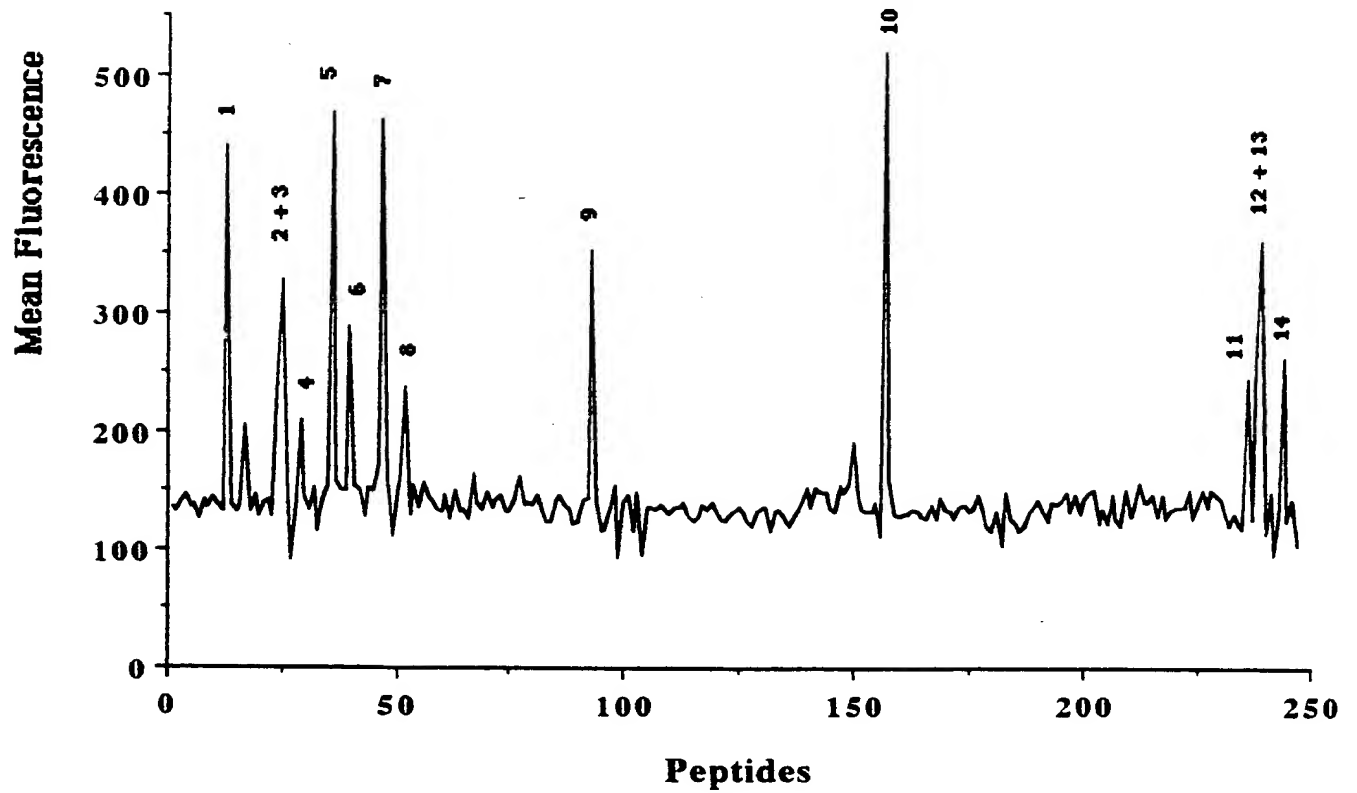
**Binding of HPV18 E6/7 peptides to HLA-A2.1**

FIG. 2



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